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(54) **A novel mutant microorganism and its use in removing organic sulfur compounds.**

(57) A novel mutant microorganism Pseudomonas sp. CB1 having a registry number ATCC 39381 has been produced by chemical mutagenesis and is effective in removing organic sulfur compounds from carbonaceous materials such as fossil fuels e.g., coal, petroleum and petroleum products.

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A NOVEL MUTANT MICROORGANISM AND ITS USE IN REMOVING ORGANIC SULFUR COMPOUNDS

Background

Many grades of coal and petroleum contain large quantities of sulfur compounds which form corrosive air and water pollutant products during combustion. A number of chemical and physical processes have been developed to remove inorganic sulfur components, such as sulfates and iron pyrites. Some organic sulfur compounds, such as mercaptans, aliphatic sulfides and disulfides are relatively amenable to chemical removal. However, the aromatic sulfur compounds, such as diphenylsulfide, benzothiophene, dibenzothiophene and the like, are found in predominating concentrations in coal. These sulfur compounds are resistant to chemical and microbial attack and are frequently characterized as refractory organosulfur compounds.

A number of chemical processes for removal of organic sulfur are under investigation. Among the processes being evaluated on a laboratory or pilot scale are oxydesulfurization, chlorinolysis, oxidation, hydrodesulfurization and Gravimelt. According to Berry [Berry, R. I. (1981), "Guide to Coal-Cleaning Methods," Chemical Engineering, January 26], projected total product costs from bench and pilot scale operations ranged from \$41 to \$58 per ton (in 1979 dollars) for 10 to 50% organic sulfur removal.

Removal of such refractory organic sulfur compounds by microbial treatment would have many important advantages. Since high temperature, high pressure or corrosion resistant equipment are not required for the biological process, inexpensive construction materials can be used leading to low capital costs. Processing costs can also be low if waste materials are utilized to support the microbial growth. Biological treatment can be carried out under ambient conditions in many locales. Microbial treatment is not likely to significantly alter the structure and composition of the coal or to substantially reduce the BTU value of the coal.

Microbiological treatment of petroleum and coal has been under experimental investigation utilizing a variety of microorganisms, including genera such as Pseudomonas, Alcaligenes, Bacillus, Desulfovibrio, Thiobacillus, Arthrobacter, Flavobacterium, Beijerinckia, Rhizobium, and Acinetobacter. Some of these microorganisms show potential for degrading refractory organic sulfur compounds such as discussed by Hedrick et al. [Hedrick et al. - (1982), "Desulfurization of Coal by Biological Pretreatment," State-of-the-Art]. This article provides a comprehensive summary of such experimental efforts. However, neither this report nor

other available art discloses the mutant microorganism Pseudomonas sp. CB1 (ATCC #39381) or its efficacy in removing sulfur from refractory organic sulfur compounds, such as the thiophenes found in carbonaceous materials.

Summary

Pseudomonas sp. CB1, ATCC #39381, is a non-motile, gram negative rod approximately 0.5 by 1 micron in size, occurring singly or in short chains. It grows aerobically; produces a yellow-green water soluble pigment; is catalase and oxidase positive; does not utilize citrate as a sole carbon source; does not grow at 41°C; and grows on MacConkey's agar. Colonies formed on nutrient agar are round, flat, opaque and produce a yellow-green pigment. Colonies formed on MacConkey's agar are round, regular and white. This organism does not use naphthalene, phenol or resorcinol as a sole source of carbon for growth and energy. However, the organism is able to use benzoate as a sole carbon source. The organism contains a large (≥25 megadaltons) plasmid. A culture of this microorganism has been deposited in ATCC and has received the number 39381.

The organism was developed by adaptation of natural organisms to dibenzothiophene (DBT) and selection of the adapted culture for those organisms able to use benzoate as a sole carbon source followed by selection for organisms capable of oxidizing DBT in an aqueous medium. Organisms capable of oxidizing DBT in an aqueous medium were treated by a mutagen to increase DBT oxidation capability.

The new mutant microorganism, CB1 (ATCC #39381), successfully degrades refractory aromatic and aliphatic organic sulfur compounds and is especially useful in oxidizing and removing these contaminants from carbonaceous substrates such as coal, petroleum and petroleum products without utilization of the carbonaceous material.

The new CB1 microorganism can be used to remove organic sulfur from different grades of coal of various particle sizes from fine to coarse. It can also be used to remove organic sulfur compounds in coal/water fuel slurries and in pipeline slurries.

Detailed Description

Isolation. Soil samples were obtained in Maryland, Virginia, and from a Pennsylvania coal mine. Microbial populations were isolated from each sam-

ple by extraction with modified Ringer's solution, a sterile salts solution containing sodium chloride - (0.7%), CaCl_2 (0.0026%), KCl (0.035%), and distilled water. Each of the salts extracts containing the microorganisms was plated on nutrient agar and inoculated into Trypticase Soy Broth (TSB) for growth of the cultures. A pure culture of *Pseudomonas putida* was also inoculated into Trypticase Soy Broth. Adaptation. All of the above microbial cultures were individually adapted to the presence of DBT in Trypticase Soy Broth, and then to the presence of DBT in minimal salts medium (0.02% MgSO_4 , 0.02% citric acid $\cdot \text{H}_2\text{O}$, 1.0% K_2HPO_4 , 0.35% $\text{Na}(\text{NH}_4)\text{HPO}_4$, pH 7). The concentration of the DBT ranged from 100 to 1000 mg/L. Selection. All of the cultures which had successfully adapted to the presence of DBT (Maryland, Virginia and Pennsylvania soil populations and the *Ps. putida* pure culture) were selected for utilization of benzoate as a carbon source by inoculation into minimal salts medium (no citrate) containing only benzoate (0.25%) as the sole carbon source.

All cultures able to use benzoate as a sole carbon source were grown in minimal salts medium containing 0.25% benzoate on a shaking table at room temperature overnight. To evaluate the cultures for oxidation of DBT in aqueous medium, each overnight culture was inoculated with DBT to a final concentration of 200 or 400 mg/L. Media controls were also inoculated with DBT at the same DBT concentrations. After DBT inoculation, the cultures were returned to the shaking table and incubated for several days. To determine the amount of DBT remaining in the aqueous medium, the entire contents of each flask were extracted with methylene chloride. Three of the cultures tested - (the Maryland and Virginia soil population and the pure culture of *Ps. putida*) showed small decreases in solvent extractable DBT concentration. These cultures were maintained separately and as a combination of the three cultures. This combined culture was designated "Combo."

The three individual cultures and "Combo" were further adapted to the presence of DBT and selected for their ability to use benzoate as a sole carbon source.

Mutagen Treatment. Each of these four cultures and the Pennsylvania coal mine cultures were subjected to diethyl sulfate (DES) mutagenesis as described by Roth [Roth, J.R. (1971), "Genetic Techniques in Studies of Bacterial Metabolism: In *Methods in Enzymology*, XVII. A, p.3-35]. Some of the microorganisms in each of the five cultures survived the DES treatment and were "rescued" by serial dilution (1/100) into fresh nutrient broth for overnight quiescent growth at 35°C.

The microbial cultures resulting from "rescue" in nutrient broth following DES mutagenesis were

inoculated into minimal salts medium containing benzoate as a carbon source and grown overnight on a shaking table at room temperature. The culture flask and media control flasks were each inoculated with DBT at varying concentrations (e.g., 100 mg/L, 200 mg/L). The flasks were returned to the shaking table and incubated for varying periods of time (e.g., 18 hours, 4 days, 7 days) prior to extraction of the entire contents of the flask with methylene chloride for DBT analyses. The "Combo" culture was the only one which showed a significant decrease in solvent extractable DBT.

The "Combo" culture following the DES treatment was subcultured into minimal salts medium containing benzoate. This subculture was streaked for isolation onto nutrient agar plates. Single colony isolation was performed three times and from the third isolation, a single colony was subcultured into minimal salts medium plus benzoate and evaluated for DBT oxidation. This triple single colony isolate was designated CB1 and identified as a *Pseudomonas* sp. Plasmid Analysis. A variety of chemical agents, such as acridine orange, as well as growth at elevated temperatures are able to free or "cure" some bacterial cells of plasmid DNA molecules. Plasmids which exist as autonomously replicating circular DNA duplexes are eliminated by these agents either because of interference with replication (acridines) or by alteration of their membrane attachment sites (elevated temperatures).

The curing procedure used with CB1 was performed in accordance with the one disclosed in the "Manual of Methods for General Bacteriology" - [Gerhardt, P. (1981), *Manual of Methods for General Bacteriology*, American Society for Microbiology, Washington, D.C.]. The CB1 culture in the logarithmic growth phase was inoculated at 10^3 to 10^4 cells into a series of tubes containing nutrient broth with 50 or 250 mg/L acridine orange. Cultures were incubated overnight with aeration at 25°C or at 40°C. A portion of each culture was then diluted 1:100 into fresh medium and allowed to grow at 25°C or 40°C with no aeration. Appropriate dilutions of the culture were plated on nutrient agar to obtain single colonies. Single colony isolates were tested for DBT oxidation.

Stock cultures of CB1 (ATCC #39381), *Ps. putida* and two acridine orange treated single colony isolates which showed decreased DBT oxidation, were streaked onto nutrient agar plates and treated for isolation of plasmid DNA using the methods of Birnboim and Doly [Birnboim, H.C. and Doly, J., "A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA," *Nucleic Acids Research*, 7:1513-1523] and "lysis in the well" techniques of Newland *et al.* [Newland, J.W. *et al.* (1980), "Rapid Screening for Plasmids in Environmental Isolates of *Vibrio cholerae* by an "in

the well" Lysis Technique Using Horizontal Gel Electrophoresis," Abstracts Proc., 3rd Annual Mid-Atlantic Regional Extrachromosomal Elements Meeting, Plasmid, 3:238].

These plasmid analytic procedures demonstrated that "uncured" CB1 ATCC #39381) contained a large plasmid ≥ 25 megadaltons in size. Untreated Ps. putida, one of the parent strains in the original combination culture, did not contain any plasmids detectable by the procedures. A treated CB1 isolate, which no longer oxidized DBT following acridine orange treatment, was also analyzed and found to contain a large plasmid similar in molecular weight to the plasmid found in CB1. In this case, the acridine orange treatment apparently affected the microbial genetic material without eliminating the plasmid. The foregoing results indicated that oxidation of DBT by CB1 is not plasmid mediated. Biochemical Characterization. Biochemical characterizations of CB1, stock cultures of Ps. aeruginosa, Ps. putida and Ps. stutzeri were performed using the API 20E System [API 20E System Analytical Profile Index, Analab Products, Plainview, NY]. Profiles for CB1 and the closely related Pseudomonas species are presented in Table 1. It will be noted that important differences between CB1 and each of the other Pseudomonas are demonstrated.

Mechanistic Studies With DBT. Radioisotope studies in which CB1 was incubated with ^{14}C -DBT and ^{35}S -DBT indicated that the DBT sulfur and carbon skeleton were oxidized to water soluble compounds. However, no $^{14}\text{CO}_2$ was produced indicating that CB1 did not utilize the DBT carbon skeleton as a carbon source. The biomass was removed by centrifugation and filtration through a 0.2 micron filter. The filtrate was analyzed for sulfate as described in the Hach Handbook for Water Analysis [Hach Chemical Company, 1979] and by ion chromatography. The two analytical methods gave comparable results. Sulfate concentrations in the CB1 treated DBT medium were 100 and 99 mg/L. Sulfate concentrations in the controls were 55 and 67 mg/L. These data indicate that CB1 acts on DBT by oxidation of the sulfur group to produce inorganic sulfate. This mechanism differs from all other literature reports of microbial DBT degradation where the major product is reported to be 3-hydroxy-2-formyl benzothiophene [Kodama, K. et al. (1973), "Identification of Microbial Products from Dibenzothiophene and Its Proposed Oxidation Pathway," Agr. Biol. Chem., 37(1), p. 45-50].

Mechanistic Studies with Coal. Organic sulfur in coal is determined by a

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Table 1. API 20 E SYSTEM TEST

API Test	Organism			
	<i>Ps. aeruginosa</i>	<i>Ps. putida</i>	CBl	<i>Ps. stutzeri</i>
ONPG	-	-	-	-
ADH	+	+	-	-
LDC	-	-	-	-
LDC	-	-	-	-
ODC	-	-	-	-
CIT	+	-	-	-
HS	-	-	-	-
URE	+	+	-	-
TDA	-	-	-	-
IND	-	-	-	-
VP	-	-	-	-
GEL	+	-	-	-
GLU		-	-	-
man	-	-	-	-
ino	-	-	-	-
sor	-	-	-	-
rha	-	-	-	-
sac	-		-	-
mel	-	-	-	-
amy	-	-	-	-
ara	-	-	-	-
oxi	+	+	+	+
NO	-	-	-	+
N gas		-	-	-

Motility	+	+	-	+
MAC	+	-	+	-
OF-O	+	+	+	+
OF-F	-	-	-	-
41 C	+	+	-	+
phenol			-	
resorcinol			-	
benzoate			+	-

round-about method which involves analysis for pyritic sulfur, sulfate sulfur and total sulfur. The organic sulfur is determined by the difference between the total sulfur and the sum of the pyritic sulfur and the sulfate sulfur. To prove that CB1 acts only on the organic sulfur in coal, the coal was washed to remove the sulfate sulfur. Decrease in the total sulfur content of the washed coals ranged from 8 to 34% depending on coal type and particle size. To prove that CB1 does not act on pyritic sulfur, the following experiments were performed.

CB1 was incubated with 0.25 g iron pyrite - (approximately 325 mesh)/100 mL water on a shaking table at 25°C. A control flask contained only minimal salts medium, benzoate and iron pyrite. Following the incubation, the contents of each flask were centrifuged and the supernatant removed and filtered through a 0.2 micron filter. The filtered supernatant was analyzed for sulfate using the method described in the Hach Handbook for Water Analysis [Hach Chemical Company, 1979]. The pyrite/biomass harvested from the centrifugation was dried at 60°C overnight and analyzed for total sulfur content using a Fisher total sulfur analyzer. The total sulfur contents of the control and experimental pyrite (treated with CB1) were identical. These results indicate that CB1 does not oxidize iron pyrite. Therefore, it can be concluded that only

the organic sulfur forms in coal are oxidized by CB1.

Example 1

The oxidation of DBT from aqueous medium by the untreated "Combo" microbial culture and the mutant organism CB1 was evaluated under varying conditions of incubation time, DBT concentration and temperature. Results of these experiments, presented in Tables 2 and 3, demonstrate that CB1 oxidized significantly greater quantities of DBT in the aqueous medium than the untreated parent culture.

Example 2

The ability of CB1 to oxidize other organic sulfur compounds in an aqueous medium was evaluated in a series of studies. CB1 cultures were inoculated into minimal salts medium containing benzoate (0.25%) for overnight growth on a shaking table at 25°C. A specified concentration of a selected organic sulfur compound was added to a flask containing an overnight culture of CB1.

Table 2. Comparison of the Ability of CBl and the Parent Culture (Combo) to Oxidize DBT

Culture	Incubation Time	Initial DBT	
		Conc. mg/L	% Decrease in DBT
Combo	3 days	50	4
Combo	5 days	50	3
Combo	7 days	100	7
Combo	7 days	200	0
Combo	10 days	200	10
Combo	10 days	100	7
CBl	18 hours	100	62
CBl	18 hours	100	65
CBl	18 hours	100	60

Table 3. The Effect of Concentration and Temperature On DBT Oxidation by CBl

Incubation Time	Incubation Temperature (°C)	Initial DBT	
		Conc. mg/L	% Decrease in DBT
18 hr	25	200	71
72 hr	25	200	88
18 hr	25	250	46
18 hr	25	428	67
18 hr	25	800	44
18 hr	25	800	58
18 hr	35	250	83
18 hr	35	200	94
18 hr	40	200	76

Flasks containing the microbial culture and the organic sulfur compounds were incubated quiescently for 24-48 hours prior to extraction of the

entire contents of the culture flasks for the organic sulfur form. The contents of each flask were extracted three times with 10 mL methylene chloride

and the extract was analyzed by gas chromatography. Media control flasks contained minimal salts, benzoate and the specified concentration of the organic sulfur compound to be evaluated. A second set of media control flasks were dosed and extracted immediately to determine the extraction ef-

iciency of each organic sulfur compound. Data presented in Table 4 demonstrate that the mutant microorganism, CB1, removed significant quantities of solvent extractable organic sulfur compounds from aqueous medium.

Table 4. Oxidation of Other Organic Sulfur Compounds by CB1

Compound	Incubation Initial		
	Time	Conc mg/L	% Reduction
n-octyl sulfide	24 h	100	44
	24 h	200	85
	24 h	200	81
Benzyl methyl sulfide	24 h	100	31
	24 h	100	32
Thioanisole	24 h	100	45
	24 h	100	51
1-benzothiophene	24 h	200	81

Example 3

Studies were performed to determine the ability of the mutant organism CB1 to remove sulfur from different coal samples and different coal particle sizes. CB1 cultures were inoculated into minimal salts medium containing benzoate (0.25%) for overnight growth on a shaking table at 25°C. Powdered coal was added to each 18-24 hour microbial culture at 5% (w/v). Flasks containing the microbial culture/coal mixture were replaced on the shaking table and incubated for 24-48 hours prior to removal of the coal by centrifugation, washing of the

coal with distilled water and analysis of the coal for total sulfur.

It should be noted that results of these experiments are given in terms of total sulfur because no analytical method is currently available to accurately determine the organic sulfur content of coal. Wentz coal and Montcoal were selected for testing with CB1 because organic sulfur forms are reported to make up approximately 90% of the total sulfur content of these coals. Data in Table 5 demonstrate that treatment of the coal samples with the mutant microorganism, CB1, resulted in a significant decrease in the total sulfur content of the coal when the particle size is 250 micron (60 mesh) or less.

Table 5. Coal Desulfurization Studies

Coal Sample	Particle Size (micron)	Total Sulfur (%)	Organic Sulfur (%)	% Reduction in Total Sulfur
Wentz	245 - 75	0.67	0.6	16
				22
Wentz	75	0.67	0.60	21.5
				25
Montcoal	245	0.80	0.72	22
				21
Montcoal	80% <u>></u> 600 micron	0.80	0.72	-

Example 4

Experiments were performed to evaluate the desulfurization capability of CB1 when used with high sulfur coals. CB1 cultures were inoculated into minimal salts medium containing benzoate - (0.25%) for overnight growth on a shaking table at 25°C. Powdered coal was added to each 18 to 24 hour microbial culture at 10% (w/v). Flasks containing the microbial culture/coal mixture were replaced on the shaking table and incubated for 24 hours prior to removal of the coal by centrifugation.

25 Total sulfur analyses were performed on untreated dried coal from each coal sample, on coal samples washed for 24 hours with the minimal salts media - (no microorganisms) and on coal samples treated with CB1. Data in Table 6 illustrates organic and total sulfur removal from Sewickeley and Peabody Illinois #6 coal samples by treatment with CB1.

30 While the present invention has been described by specific embodiments thereof, it should not be limited thereto since obvious modifications will occur to those skilled in the art without departing from the spirit of the invention or the scope of the claims.

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Table 6. Desulfurization of Sewickeley and Peabody Illinois #6 Coal

Sample	Total Sulfur (%)	Decrease In Total Sulfur	Decrease in Organic Sulfur
Sewickeley control (325 mesh)	5.1		
washed	4.1		
treated	3.1	24%	53%
Sewickeley control (325 mesh)	4.95		
washed	4.28		
treated	3.56	17%	48%
Peabody control (-60 mesh)	3.4		
washed	3.3		
treated	2.6	21%	53%
Peabody control (-270 mesh)	3.8		
washed	3.6		
treated	2.36	34%	93%

APPROXIMATE ANALYSIS (%)

Sewickeley coal

pyritic sulfur	2.1
sulfate sulfur	1.0
organic sulfur	1.9
total sulfur	5.0

Peabody coal

pyritic sulfur	2.07
sulfate sulfur	0.5
organic sulfur	1.33
total sulfur	3.5

Claims

1. A biologically pure culture of mutant
Pseudomonas sp. CB1 ATCC #39381.

2. A process for removing organic sulfur compounds from a carbonaceous substrate comprising treating said substrate with a culture of Pseudomonas sp. CB1 ATCC #39381.

3. The process of claim 2 in which the substrate is coal.

4. The process of claim 2 in which the substrate is petroleum and products thereof.

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European Patent
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EUROPEAN SEARCH REPORT

Application number

EP 85 11 2450

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	JP-A-60 041 479 (ATLANTIC RESEARCH CORP.) * Whole document * & US - A - 4 562 156 (Publ. 31-12-1985) -----	1-4	C 12 N 1/20 C 12 N 15/00 C 10 G 32/00 C 10 L 9/02 (C 12 N 1/20 C 12 R 1:38)
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
			C 12 N C 12 R C 10 G C 10 L
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 28-05-1986	Examiner DESCAMPS J.A.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	